THE ENERGY-TRANSFORMING STEP IN NITROBACTER-CHEMOSYNTHESIS*

by

LUTZ KIESOW,

BIO-ENERGETICS LABORATORIES

Naval Medical Research Institute

Bethesda, Maryland

Md.

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SUMMARY

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IN CHEMOSYNTHESIZING HOMOGENATES OF NITROBACTER WINOGRADSKYI THE FORMATION OF REDUCED PYRIDINE-NUCLEOTIDE (DPN) WAS OBSERVED DURING THE OXIDATION OF THE SUBSTRATE, NITRITE. OVER THE TESTED RANGE FROM pH 6.8 TO pH 9.2 THE KINETICS OF THE TWO REACTIONS ARE pH-DEPENDENT IN IDENTICAL MANNER. THE TWO REACTIONS, EQUALLY SENSITIVE TO INHIBITION BY BOTH, 1×10^{-3} MOLAR O-PHENANTHROLINE OR 5×10^{-4} MOLAR KCN, MUST THEREFORE BE COUPLED. THEY ENABLE THE CHEMO-AUTOTROPHIC MICROORGANISM TO HARNESS THE ENERGY LIBERATED IN THE SUBSTRATE OXIDATION AND TO MAKE IT AVAILABLE FOR THE SYNTHETIC PROCESS THAT ASSIMILATES THE CARBON DIOXIDE.

AUTHOR

I. INTRODUCTION

At least three methods are available today for measuring the assimilation of carbon dioxide in the chemosynthetic, nitrifying microorganism Nitrobacter winogradskyi:

1) Manometric CO_2 DETERMINATION (4)
2) Determination of the change in hydrogen ion-concentration in the bicarbonate CO_2 buffer system (5)
3) The observation of incorporation and distribution of carbon-dioxide labelled with the radioisotope ^{14}C (see figure 1).

These three methods, while of different usefulness for different aspects of the problem, lead invariably to one result, namely:

The processes of chemo-autotrophic carbon assimilation are quantitatively dependent on the energy from oxidation of an inorganic compound. For Nitrobacter this is the oxidation of nitrite-ions to nitrate-ions. As shown by figure 1 no fixation of radioactive CO_2 takes place without this reaction. The stoichiometric connection was first demonstrated 1916 by Otto Meyerhof (7) who

proved experimentally the following formulation for the balance of the reaction:

$$2\ NO_2^- + O_2 = 2\ NO_3^-$$

Today there is hardly any doubt that Meyerhof's equation describes correctly the balance of nitrite-oxidation under certain conditions. However, it is also obvious that Meyerhof's equation disregards the individual steps in which the free energy-change of nitrite-oxidation is harnessed and made available for the process of carbon dioxide assimilation.

In the search for further reaction products of nitrite-oxidation Aleem and Nason (1) believe they have found the solution in a synthesis of adenosine-triphosphate (ATP) coupled with nitrite-oxidation. Using radioactive phosphate the authors demonstrated that particulate fractions of Nitrobacter agilis incorporate inorganic phosphate into ATP in a reaction which depended upon the presence of nitrite. The optimal P/O quotient which they obtained in these experiments was 0.2.

However, it is a fact of cell physiology that Nitrobacter is capable of producing ATP (6) through "residual

respiration" (2) in the absence of nitrite. Under aerobic conditions this respiration proceeds with a Q_{O_2} of -6 (mm3 O_2/mg dry weight of cells per hour) at 30 °C. It maintains in the cells a steady-state level of 10^{-3} molar ATP (6). The P/O quotient of this respiration is approximately 3.0 (6). Furthermore it has been demonstrated that under otherwise identical conditions the addition of nitrite leads to a reduction, not to an increase of the intracellular level in ATP concentration (6). This would suggest that ATP-consuming rather than ATP-producing reactions are accelerated or initiated upon the addition of nitrite, thus leading to an increased turnover of labelled phosphate as observed by Aleem and Nason.

Looking again, with the knowledge of these facts, at Figure 1 one could hardly accept the hypothesis that nitrite-coupled synthesis of ATP is the reaction which permits the assimilation of carbon-dioxide by chemo-autotrophic cells dependent, as the figure shows, on the presence of nitrite. For, although in aerobiosis ATP is available to the cells under either conditions, the cells assimilate carbon-dioxide only in the presence, and not in the absence of nitrite. Therefore, a different reaction mechanisms must make it possible for nitrite-oxidation to perform the assimilation of CO_2.

II. NITRITE OXIDATION AND PYRIDINE-NUCLEOTIDE REDUCTION

Since the assimilation of carbon-dioxide by autotrophic cells, whatever its detailed steps may be, is in essence a reduction of CO_2, it was suggestive to look for a participation of the classic oxidation reduction-coenzymes, the pyridine nucleotides, in Nitrobacter-chemosynthesis. While in the work with nitrifying chemoautotrophs the spectrophotometric method lends itself to the determination of pyridine-nucleotide reduction, a problem of this method must first be solved, which arises from two special characteristics of the nitrite ion:

1. The ultraviolet absorption-maximum at 355 m μ interferes with that of reduced pyridine-nucleotide.

2. Nitrite has a quenching effect on the fluorescence of pyridine-nucleotide.

For these two reasons neither the direct optical nor the fluorometric method for pyridine-nucleotide reduction is applicable when simultaneously, an oxidation of nitrite takes place. To circumvent this difficulty we

introduced the interception of reduced pyridine-nucleotide by means of pyruvate and lactate-dehydrogenase in an "enzymic-trap":

$$CH_3CO\text{-}COOH + DPN\text{-}H + H^+$$

LACTATE - DEHYDROGENASE

$$CH_3CHOH\text{-}COOH + DPN+$$

Thereby it became possible to follow the reduction of pyridine-nucleotide by analyzing the lactate.

It is demonstrated with figure 2 that in homogenates of Nitrobacter a formation of DPNH takes place, dependent on the oxidation of nitrite. From table 1 it follows under what special conditions the nitrite-oxidation-coupled reduction of DPN may be observed. These special conditions include the following three:

(1) Inhibition with 5×10^{-4} molar KCN
(2) Inhibition with 1×10^{-3} molar o-phenanthroline
(3) pH dependence of reaction rate with identical optima for DPN-reduction and nitrite oxidation.

These observations prove not only the enzymic nature of the DPN reduction. They prove that DPN reduction is coupled with nitrite oxidation, as both reactions have

the observed characteristics or prerequisites in common.

III. DISCUSSION

Whereas no doubt is left by the experiments that reduced diphosphopyridine-nucleotide is a reaction-product of nitrite oxidation, difficulties are encountered in fully understanding this reaction. While Meyerhof's equation (1) for nitrite oxidation may be reformulated as

$$NO_2^- + H_2O \rightleftharpoons NO_3^- + 2H^+ + 2e \qquad (2)$$

and the formation of DPNH as

$$DPN^+ + 2H^+ + 2e \rightleftharpoons DPNH + H^+ \qquad (3)$$

a thermodynamic problem arises immediately. The standard potentials of reaction (2) and (3) are:

2: $E' = + 0.42$ V

and 3: $E' = - 0.32$ V

With a positive free energy of the combined reactions as written, the equilibrium of the coupled reactions (2) and (3) must be decidedly on the side of the reactants, nitrite and DPN^+. Indeed, nitrate-reductase, discovered by Nason (8), is an enzyme system that oxidizes TPNH while reducing nitrate to nitrite, in a practically irreversible manner.

There are two other reasons why it is improbable that pyridine-nucleotide reduction coupled with nitrite oxidation is simply an inversion of the nitrate-reductase reaction:

(1) Triphosphopyridine-nucleotide, TPN, is not reduced by nitrite in homogenates of Nitrobacter,

(2) The reduction of DPN by nitrite is not, as one might conclude from the equations 2 and 3, an anaerobic process. Aerobically, in a cell-free Nitrobacter homogenate, at 30 °C in 20 minutes

0.43 µMole DPN-H

were formed. Anaerobically, in the same homogenate, nitrite reduced

0.00 µMole DPN-H

This experiment seems furthermore to show, how nature solved the thermodynamic problem. Oxygen participates, and is consumed in stoichiometric quantities, rather than exerting a catalytic function. An as yet unknown, oxygen consuming reaction must supply the additional energy required for the reduction of the pyridine-nucleotide.

IV. EXPERIMENTS

Homogenates of Nitrobacter winogradskyi:

Nitrobacter was cultivated for 72 hours in solution K (6) at 35 °C with the trace elements added, and harvested and washed at 10 °C in a centrifuge with continuous flow. We suspended 1 ml of packed cells in 10 ml glass-distilled H_2O and homogenized this suspension with 30 grams of glass beads #10 (English Glass Co.), diameter 0.13 - 0.20 mm, for 4 minutes under cooling with ice at 11,000 rpm in the Servall-Omni-Mixer. On a fritted glass filter-funnel the homogenate was separated from the glass beads, diluted to 50 ml with glass-distilled water and kept at 0 °C until the experiment began.

L-(+)-Lactate: was determined with the method of Warburg in the arrangement described by Hohorst (3). The cuvette of d-1 cm contained: 2.6 ml Hydrazine-Glycine-buffer, pH-8.5 (3) and 0.3 ml of the deproteinized, neutral, lactate-containing sample. After 20 minutes, during which the pyruvate reacted with the hydrazine to completion, 0.15 ml of 5×10^{-2}m DPN solution were added. After a reading of the optical

density the reaction was initiated by adding 0.02 ml lactate dehydrogenase. Fifteen minutes later, when the reaction had come to a standstill the final reading of the O.D. was taken. We have satisfied ourselves that the amounts of pyruvate present in our extract do not disturb the analysis for lactate in this procedure.

Test for the NO_2^- Dependent Reduction of DPN:

Incubation took place in Erlenmeyer flasks of 50 ml capacity at 30 °C, in a metabolic shaker, under vigorous agitation with air as the gas phase. In a total volume of 10 ml the flasks contained 600 micromoles potassium phosphate, pH 7.8, 30 micromoles DPN, 5.5 micromoles sodium pyruvate, 50 micromoles nicotinamide, 145 micromoles sodium nitrite, 0.2 mg lactate-dehydrogenase, freed of ammonium sulfate by dialysis, Nitrobacter homogenate, amount corresponding to 10 mg protein. The reaction was initiated by addition of the sodium nitrite. At the required times, samples of 2 ml were taken from the flask, and deproteinized with $HClO_4$ (final concentration, 7 Volume %). These samples were analyzed for lactate as a clear supernatant after neutralization with K_2CO_3, and subsequent centrifugation. Under these conditions the

rate of reaction was found to be strictly proportional to the amount of Nitrobacter homogenate in the assay.

Dependence of Reaction Rates on H^+-Ion Concentration in DPN-Reduction and NO_2^-- Oxidation: The experimental setup was essentially the same as in other determinations of the NO_2^--dependent DPN-reduction except that potassium phosphate buffer was replaced with 0.1 molar sodium pyrophosphate-HCl-buffer. pH of the buffer was varied over the range as shown in figure 3. NO_2^-- concentrations were determined in aliquots of the incubate according to Griess-Ilosvay (see reference 4).

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TABLE 1. REDUCTION OF DPN

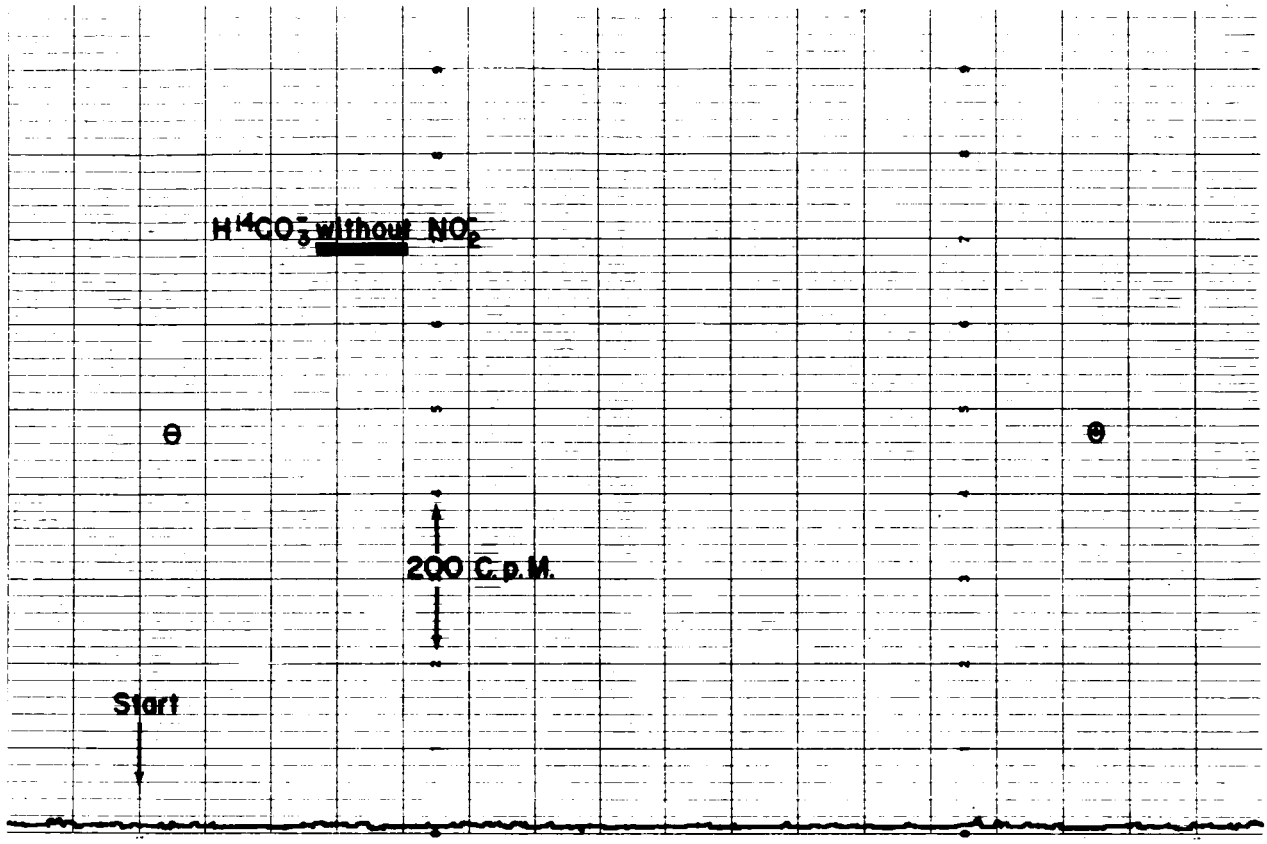
Substrates Added To Nitrobacter Homogenate In Test (Molarities)	Quantities DPN-H Found (Micromoles)
0 DPN; 0 NO_2^-	0.02
3×10^{-3} DPN; 0 NO_2^-	0.10
0 DPN; $14.5 \times 10^{-3} NO_2^-$	1.00
3×10^{-3} DPN; $14.5 \times 10^{-3} NO_2^-$	3.85

Duration of the reaction was 30 minutes at 30 °C.

Further explanations, see under 'Methods'

FIGURE 1.

THE NITRITE DEPENDENT ASSIMILATION AND DISTRIBUTION OF RADIOACTIVE CARBON IN INTERMEDIARY METABOLITES OF NITROBACTER WINOGRADSKYI $NaH^{14}CO_3$ WAS ADDED TO NITROBACTER IN A STEADY STATE OF NITRITE-OXIDATION (BELOW) OR IN THE ABSENCE OF NITRITE (ABOVE). AFTER 60 SECONDS THE CELLS WERE KILLED AND EXTRACTED WITH BOILING METHANOL. COMPOUNDS WERE SEPARATED BY IONOPHORESIS ON PAPER. AMOUNT OF EXTRACT CORRESPONDED TO 10 MICROLITERS OF PACKED CELLS (CHEMICAL IDENTIFICATION OF PEAKS WILL BE DESCRIBED ELSEWHERE).


$H^{14}CO_3^-$ without NO_2^-
200 C.p.M.
Start

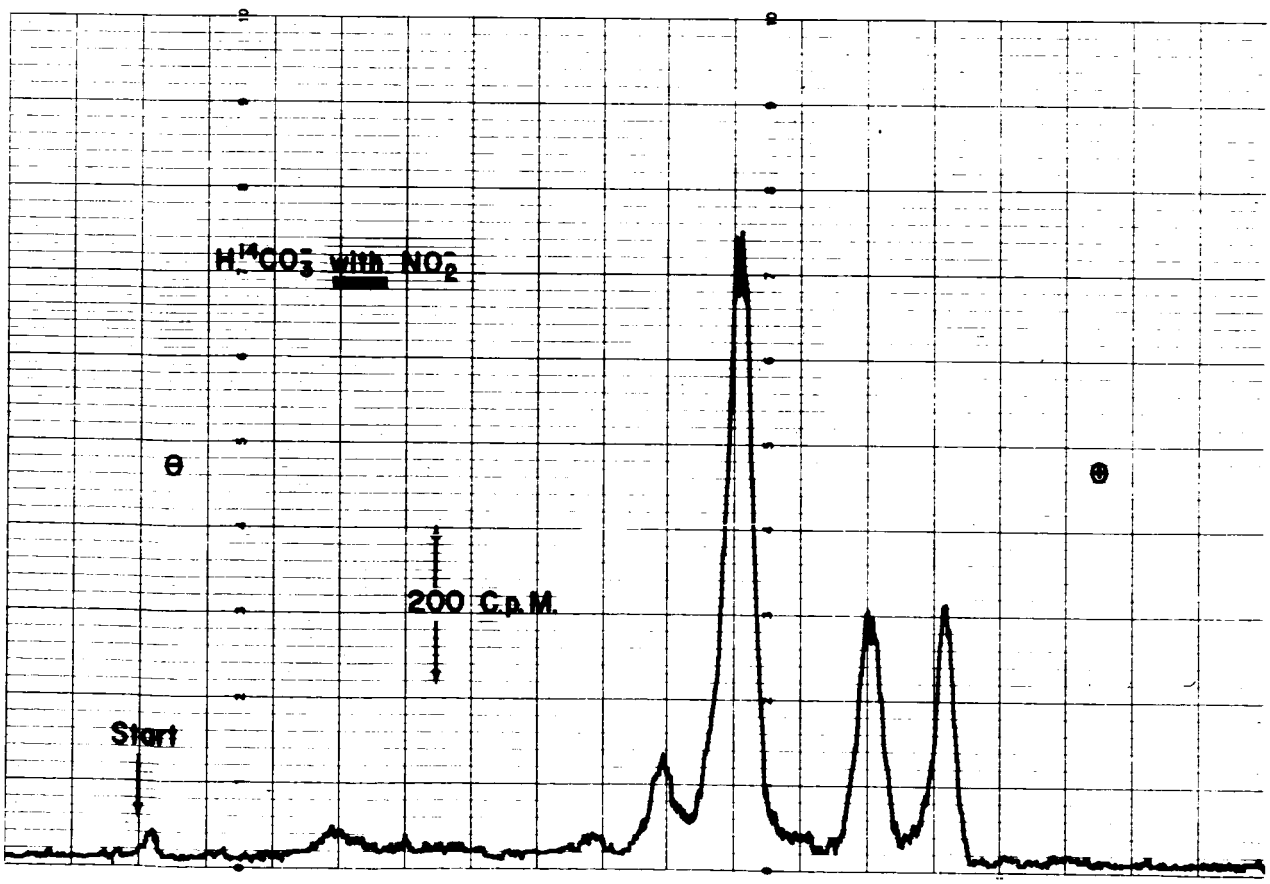

$H^{14}CO_3^-$ with NO_2^-
200 C.p.M.
Start

FIGURE 2.

THE NITRITE OXIDATION-COUPLED REDUCTION OF DIPHOSPHOPYRIDINE-NUCLEOTIDE (EXPLANATIONS, SEE UNDER METHODS)

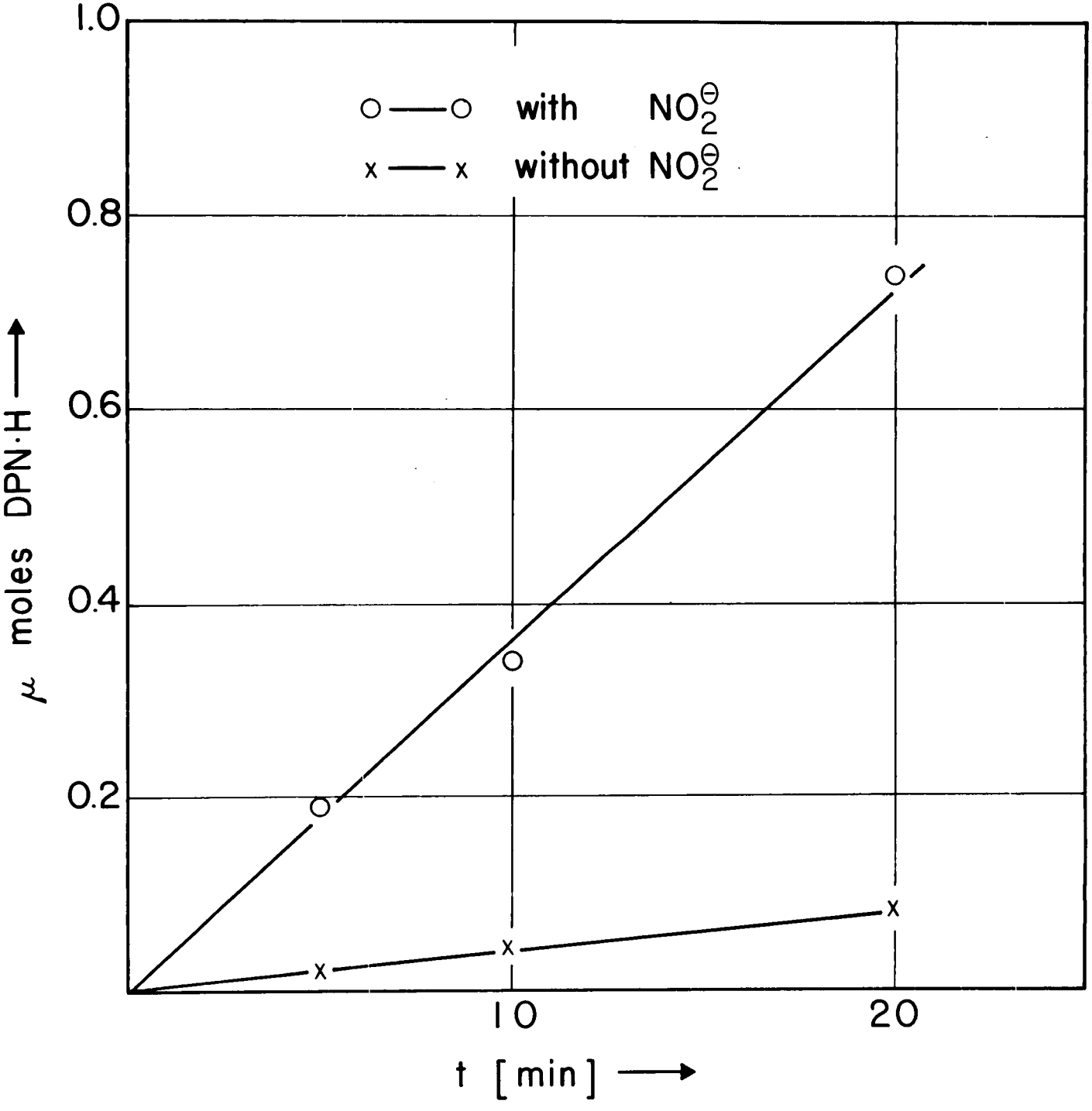
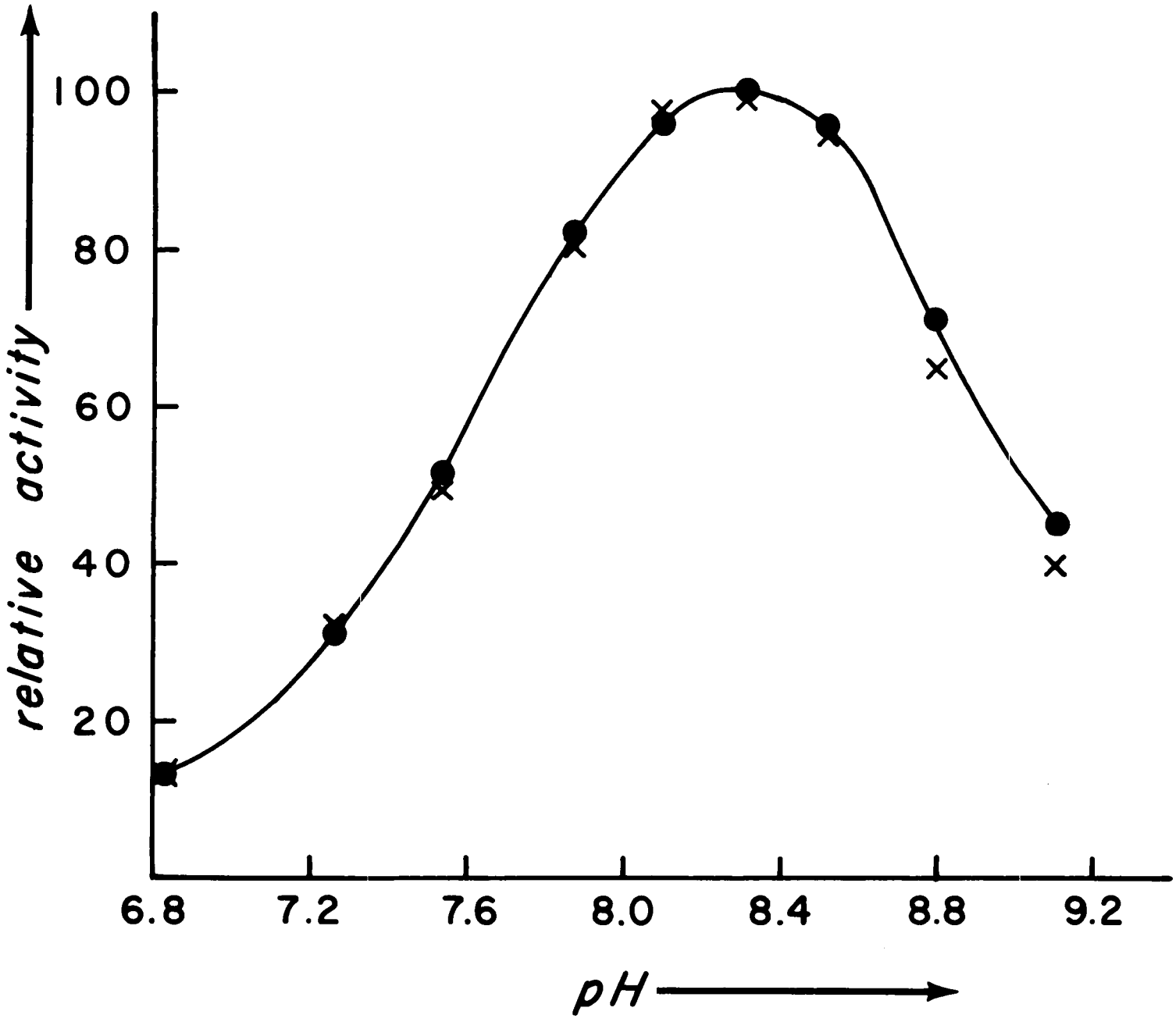

1.0
0.8
0.6
0.4
0.2
μ moles DPN·H
o—o with $NO_2^{\ominus}$
x—x without $NO_2^{\ominus}$
10
20
t [min]

FIGURE 3.

THE IDENTICAL pH-DEPENDENCE OF DPN-REDUCTION AND NITRITE-OXIDATION-RATES. CIRCLES INDICATE NO_2^--OXIDATION, CROSSES, DPN-REDUCTION.


relative activity
100
80
60
40
20
6.8
7.2
7.6
8.0
8.4
8.8
9.2
pH